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Minoxidil specifically decreases the expression of lysine hydroxylase in cultured human skin fibroblasts

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The levels of lysine hydroxylase protein and the levels of the mRNAs for lysine hydroxylase and the α- and β-subunits of proline 4-hydroxylase were measured in cultured human skin fibroblasts treated with 1 mM-minoxidil. The data demonstrate that minoxidil decreases the amount of lysine hydroxylase protein, this being due to a decrease in the level of lysine hydroxylase mRNA. The effect of minoxidil appears to be highly specific, as no changes were observed in the amounts of mRNAs for the α- and β-subunits of proline 4-hydroxylase.

INTRODUCTION

Lysine hydroxylase (procollagen-lysine,2-oxoglutarate 5-dioxygenase; EC 1.14.11.4) catalyses the formation of hydroxylysine in collagens and related proteins by the hydroxylation of lysine residues in peptide linkages [1,2]. The hydroxylysine residues formed in the reaction have two important functions. Their hydroxy groups serve as attachment sites for carbohydrate units, and they are essential for the stability of intermolecular collagen cross-links. Lysine hydroxylase is very similar to proline 4-hydroxylase (procollagen-proline,2-oxoglutarate 4-dioxygenase; EC 1.14.11.2) in its catalytic properties. Both enzymes act on non-hydroxylated collagens and collagen-like peptides, and both enzymes require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate [1–4]. However, lysine hydroxylase is an α_2 dimer consisting of only one type of subunit [1–5]. This differs from proline 4-hydroxylase, an $\alpha_2\beta_2$ tetramer with catalytic sites located mainly on the α-subunits [3,4]. The β-subunit of proline 4-hydroxylase is a highly unusual multifunctional polypeptide, being identical to the enzyme protein disulphide isomerase [6,7], a cellular thyroid-hormone-binding protein [8,9], and one of the two kinds of protein component of the microsomal triacylglycerol transfer protein complex [10]. Complete cDNA-derived amino acid sequences are now available for lysine hydroxylase [11] and the α-subunit [12,13] and β-subunit [7–9,14,15] of proline 4-hydroxylase from several species.

Minoxidil, an antihypertensive piperidinopyrimidine derivative, has been reported to suppress lysine hydroxylase activity and the proliferation of cultured human skin fibroblasts [16]. The effect appeared to be specific, as no decrease was found in proline 4-hydroxylase activity, the incorporation of [^{3}H]leucine into protein or the incorporation of [^{3}H]uridine into RNA, whereas a significant decrease was found in the incorporation of [^{3}H]thymidine into DNA [16]. The data suggested that minoxidil may suppress the synthesis of lysine hydroxylase itself or of an activator regulating lysine hydroxylase activity in the cell [16]; however, cellular levels of the lysine hydroxylase protein could not be studied because of a lack of antibodies to the enzyme.

In the present study we have measured the levels of lysine hydroxylase protein and the levels of the mRNAs for lysine hydroxylase and the two types of subunit of proline 4-hydroxylase in cultured human skin fibroblasts treated with minoxidil. Our data indicate that this compound specifically suppresses the gene expression of lysine hydroxylase at a pre-translational level.

MATERIALS AND METHODS

Cell culture

Locally established human skin fibroblasts were cultured on 100 mm Petri dishes (Falcon) in 10 ml of Dulbecco's modified Eagle's medium (Gibco). The medium was buffered to pH 7.4 with 45 mM-sodium bicarbonate and supplemented with 10% (v/v) fetal calf serum which had been inactivated overnight at 37 °C. The cells were incubated at 37 °C in an atmosphere of air/CO_2 (19:1).

The cells were first grown to confluency. The medium was then replaced with medium that contained 1 mM-minoxidil (Sigma Chemical Co.) dissolved in 95% ethanol. The final concentration of ethanol was 1% (v/v). The control medium contained 1% (v/v) ethanol without minoxidil. After the incubation, the cells were washed carefully with 0.14 M-NaCl in 20 mM-phosphate buffer, pH 7.4, and stored at −70 °C for further studies.

Western blot analysis

A polyclonal antibody against a synthetic peptide of 14 amino acids corresponding to nucleotides 1926–1967 in the human lysine hydroxylase cDNA sequence (T. Hautala, M. G. Byers, R. L. Eddy, T. B. Shows, K. I. Kivirikko, R. Myllylä, unpublished work) was produced in a rabbit. The synthetic peptide was conjugated to haemocyanin before the immunization [17].

Soluble proteins from minoxidil-treated and untreated human skin fibroblasts were analysed by immunoblotting. The washed, frozen cells (about 5×10^6 cells) were scraped with a rubber policeman into 0.25 ml of a solution containing 0.15 M-NaCl, 0.1% (v/v) Triton X-100 6.7 mM-EDTA, 28 mM-*N*-ethylmaleimide, 3.4 mM-*p*-aminobenzoic acid and 10 mM-Tris/HCl, pH 7.5, at 4 °C. The suspension was sonicated for 2×15 s and the soluble proteins were separated by centrifugation at 15000 *g* for 20 min. The protein concentration of the supernatant was determined by the method of Bradford [18]. An aliquot of the supernatant was electrophoresed by SDS/PAGE (8% gels). The fractionated proteins were transferred electrophoretically to a poly(vinylidene difluoride) membrane and incubated with the antiserum against the synthetic peptide, and the bound antibodies were visualized by peroxidase-conjugated goat anti-(rabbit IgG), using 4-chloro-1-naphthol as a substrate for the peroxidase.

Determination of mRNA levels

Total RNA was isolated from the cultured human fibroblasts

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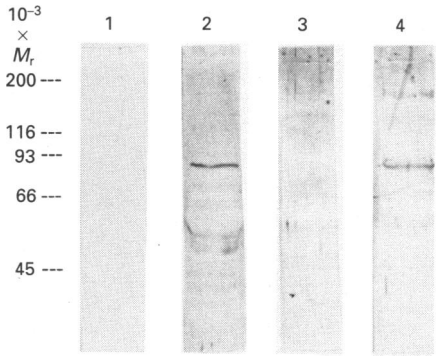

Fig. 1. Western blot analysis of soluble proteins from minoxidil-treated and control fibroblasts

Confluent human skin fibroblasts were cultured without minoxidil (lanes 1 and 4) or with 1 mM-minoxidil for 10 h (lane 2) or 96 h (lane 3). Electrophoretically fractionated soluble proteins (28 μg/lane) were transferred to nitrocellulose and immunostained as described in the Materials and methods section. The antiserum against the synthetic peptide (lanes 2–4) and preimmune serum (lane 1) were both used at a dilution of 1:3000. Positions of the M_r markers are shown.

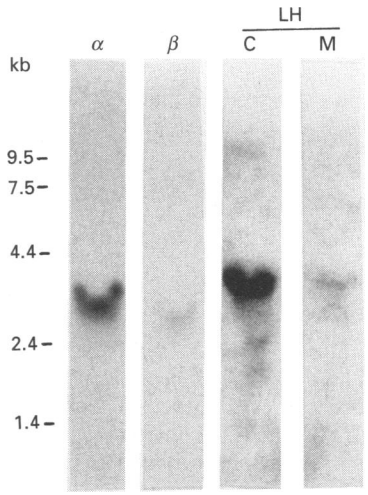

Fig. 2. Northern blot analyses of mRNA for lysine hydroxylase and for the α- and β-subunits of proline 4-hydroxylase in minoxidil-treated fibroblasts

Total RNA of confluent cultured fibroblasts was separated electrophoretically on a 0.7% agarose gel containing 2 M-formaldehyde, transferred to a nitrocellulose filter and hybridized with the labelled cDNA probes for the α- and β-subunits of proline 4-hydroxylase and for lysine hydroxylase (LH). In the case of lysine hydroxylase both minoxidil-treated (M) and untreated cells (C) are shown. The amount of total RNA used in the α and β lanes was 15 μg, whereas the amount of RNA in the LH lane was 30 μg. The positions of RNA markers are shown.

by the guanidine isothiocyanate method [19]. For Northern blot analysis the RNAs were electrophoresed in a 0.7% agarose gel containing 2 M-formaldehyde and transferred to a nitrocellulose filter. To determine the mRNA levels, serial dilutions of total RNA were dotted on to nitrocellulose filters using a vacuum manifold (Minifold II; Schleicher and Schuell). The Northern blot and slot-blot filters were hybridized under conditions of 50% formamide, 5 × SSC (1 × SSC = 0.15 M-NaCl and 15 mM-sodium citrate buffer, pH 6.8), 1% BSA, 1% Ficoll, 1% polyvinylpyrrolidone, 0.25 mg of denatured salmon sperm DNA/ml and 0.1% SDS at 42 °C overnight. The filters were washed with 0.5 × SSC/0.1% SDS at 45 °C and exposed to Kodak X-Omat film. mRNA was quantified using Molecular Dynamics Computing Densitometer Model 300A.

RESULTS

Western blot analysis of lysine hydroxylase protein levels

Western blot analysis was performed to determine whether minoxidil influences cellular levels of the lysine hydroxylase protein. Blots containing SDS/PAGE-fractionated proteins from confluent minoxidil-treated and untreated human skin fibroblasts were incubated with antiserum prepared against a synthetic peptide corresponding to amino acid residues 576–589 in a hydrophilic area of the *C*-terminal domain of human lysine hydroxylase (T. Hautala, M. G. Byers, R. L. Eddy, T. B. Shows, K. I. Kivirikko & R. Myllylä, unpublished work). An antiserum dilution of 1:3000 was found to give the best staining under the conditions used.

The antiserum stained a polypeptide with an apparent M_r of about 85000 (Fig. 1) in the soluble protein fraction of untreated human skin fibroblasts. Polyclonal antibodies prepared in a rabbit against purified chick embryo lysine hydroxylase [5] recognized the same polypeptide band (results not shown). The size of the immunostained polypeptide is in excellent agreement with the value reported for human lysine hydroxylase in SDS/PAGE [5,20] and with the value calculated from the human lysine hydroxylase cDNA sequence. Culture of cells with 1 mM-minoxidil for 4 days resulted in essentially a total loss of the immunostained polypeptide from the soluble protein fraction, whereas culture with minoxidil for only 10 h caused no detectable changes in the level of this polypeptide (Fig. 1).

Northern blot analysis

Confluent cultures of human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% ethanol or 1% ethanol containing 1 mM-minoxidil. Total cellular RNA was isolated from the minoxidil-treated and untreated cells and analysed by Northern hybridization by using as probes a cDNA clone for human lysine hydroxylase and cDNA clones for the two types of subunit of human proline 4-hydroxylase.

Each labelled cDNA probe hybridized in both the minoxidil-treated (Fig. 2) and untreated (results not shown for α- and β-subunits of proline 4-hydroxylase) cells to a single mRNA species, the sizes of which were in agreement with those reported previously ([7,12]; T. Hautala, M. G. Byers, R. L. Eddy, T. B. Shows, K. I. Kivirikko & R. Myllylä, unpublished work). However, the band hybridizing to the human lysine hydroxylase probe was much fainter in intensity in the minoxidil-treated cells than in the untreated cells (Fig. 2).

Slot-blot hybridization analysis

To quantify the changes in the levels of the mRNAs in the minoxidil-treated and untreated cells, three serial dilutions of denatured total RNA were dotted on to a nitrocellulose membrane. The slot-blot filters were then hybridized with cDNA probes for human lysine hydroxylase and the two types of subunit of human proline 4-hydroxylase (Fig. 3). A cDNA probe for human γ-actin was used as a control to verify that the changes found in the mRNA concentrations were not due to non-specific effects or artifacts. The relative mRNA levels were quantified using a densitometer.

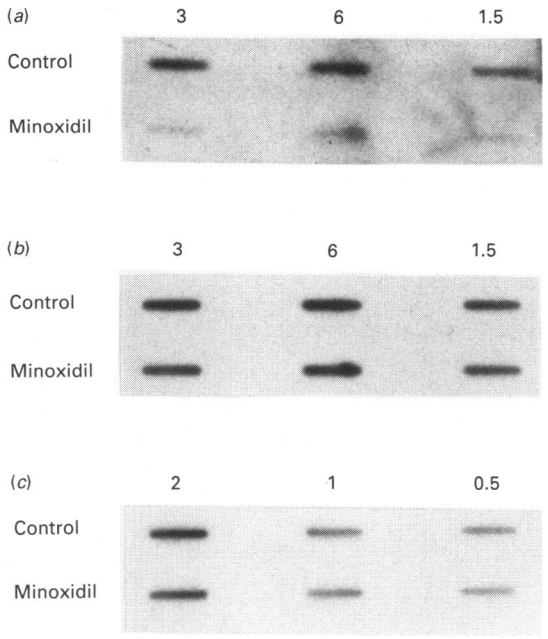

Fig. 3. Slot-blot hybridization analysis of the mRNA levels for lysine hydroxylase and for both subunits of proline 4-hydroxylase

Total RNA from minoxidil-treated and untreated (control) cells was blotted on to nitrocellulose filters at various concentrations (0.5–6 μg; values given above lanes) and hybridized with labelled cDNAs for lysine hydroxylase (*a*) and for the α- (*b*) and β- (*c*) subunits of proline 4-hydroxylase.

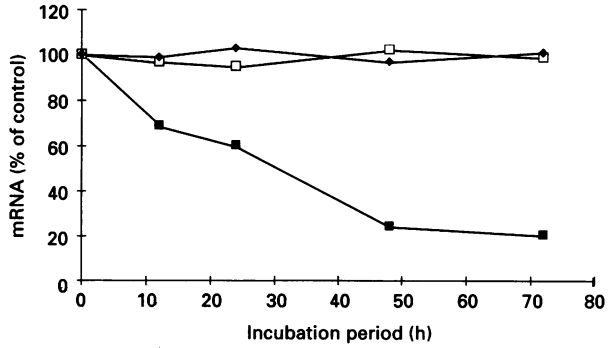

Fig. 4. Time-dependence of the effect of minoxidil on mRNA levels of lysine hydroxylase and the subunits of proline 4-hydroxylase

Confluent cultures of human skin fibroblasts were incubated in the presence of minoxidil for 12, 24, 48 and 72 h. Total RNA was isolated and blotted on to nitrocellulose filters and hybridized with labelled cDNAs for lysine hydroxylase (■) and for the α- (□) and β- (◆) subunits of proline 4-hydroxylase. The amount of mRNA was quantified on a densitometer. The values are given as percentages of the value in the corresponding untreated cells. Each time point represents the mean value from four experiments.

Exposure of the cells to 1 mM-minoxidil for 72 h resulted in a progressive decrease in the level for lysine hydroxylase mRNA, the value at 24 h being about two-thirds and at 48 h and 72 h about one-fifth of that in the untreated cells (Figs. 3 and 4). No changes were found in the mRNA levels of the α- and β-subunits of proline 4-hydroxylase (Fig. 4) or of γ-actin (results not shown) at any of the time points.

DISCUSSION

A number of previous studies have demonstrated that changes in the amount of proline 4-hydroxylase activity in cultured cells and tissues usually parallel changes in the rate of collagen synthesis [1–4]. The levels of lysine hydroxylase activity likewise often change with changes in the rate of collagen synthesis, but these levels are also dependent on the type of collagen synthesized [1,2]. Marked changes in the levels of mRNAs for the two types of subunit of proline 4-hydroxylase have recently been reported in some situations [21,22], whereas no data have been available on the mechanisms involved in the changes of the levels of lysine hydroxylase activity.

The present data demonstrate that minoxidil specifically decreases the amount of lysine hydroxylase protein in cultured human skin fibroblasts. The previously reported decrease in lysine hydroxylase activity caused by minoxidil [16] is thus not due to a decrease in the activity of the lysine hydroxylase protein or to a change in the synthesis of some protein regulating the amount of lysine hydroxylase activity. The present data further demonstrate that minoxidil markedly decreases the amount of lysine hydroxylase mRNA, thus indicating that the decrease in the amount of lysine hydroxylase protein is due to a decrease in its synthesis at a pretranslational level. The decrease in the lysine hydroxylase mRNA appeared to be highly specific, as no changes were seen in the amounts of mRNAs for the α- or β-subunits of proline 4-hydroxylase or for γ-actin.

The data do not indicate the mechanisms leading to the decrease in the amount of the lysine hydroxylase mRNA. The high specificity of the marked decrease makes it likely that it is due to a decreased transcription of the lysine hydroxylase gene. Nevertheless, collagen mRNA concentrations are also regulated at the level of mRNA stability [23,24], and a decreased mRNA stability may well contribute to or be responsible for the changes found here. Genomic clones containing the promoter region of the human lysine hydroxylase gene are not yet available. Such clones will make it possible to initiate detailed studies on the mechanisms regulating the transcription of the human lysine hydroxylase gene.

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